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Systemic infection induced by Campylobacter jejuni: Development of a mouse model and elucidation of molecular mechanisms

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Systemic infection induced by *Campylobacter jejuni*: Development of a mouse model and elucidation of molecular mechanisms

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ABSTRACT

Campylobacter jejuni clone SA has emerged as the predominant cause of *Campylobacter*-associated ovine abortion in the U.S., and this clone is highly pathogenic in pregnant sheep and guinea pigs. To induce abortion, orally ingested *Campylobacter* must be able to translocate across the intestinal epithelium and spread systemically. To understand the pathogenic mechanisms and immune protection of *C. jejuni*-induced abortion, it is necessary to develop a cost-effective animal model to evaluate systemic infection induced by this pathogenic clone. In this study, two different breeds of female mice (BALB/c and CD-1) were orally inoculated with *C. jejuni* IA3902, a clinical clone SA isolate whose complete genome sequence has been determined, to evaluate the induction of bacteremia and hepatic infection. Our results revealed that CD-1 mice were more susceptible than BALB/c mice to infection by IA3902. In CD-1 mice, *C. jejuni* IA3902 induced bacteremia and hepatic infection within 1 hour after oral inoculation, and bacteremia peaked at 8 and/or 12 hours after inoculation. Compared with IA3902, the magnitude and duration of bacteremia and hepatic infection induced by *C. jejuni* strains NCTC 11168 and 81-176 were significantly less prominent, indicating that IA3902 is more virulent than the other strains tested with regard to systemic spread. Mutagenesis in IA3902 showed that the loss of the capsule (Δ*kpsS*) completely prevented the organism from causing bacteremia and hepatic infection, while the loss of pVir plasmid did not affect systemic spread. These findings indicate that the CD-1 mouse model is suitable for examining critical steps of *Campylobacter* pathogenesis and identify the capsule as a key virulence factor of this pathogenic organism to induce bacteremia.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Campylobacter jejuni has long been recognized as a cause of bacterial foodborne illness, and it remains the most prevalent bacterial foodborne pathogen in the industrial world to date (38). In 2009, according to the Centers for Disease Control and Prevention (CDC) FoodNet surveillance program, *Campylobacter* was identified as the second leading cause of laboratory-confirmed bacterial foodborne disease (13.02 per 100,000 people) behind *Salmonella* (15.19 per 100, 000 people) in the United States (32). In fact, the latest estimate from the CDC indicated 800,000 cases of campylobacteriosis occur annually in the United States (32).

Several factors have been found to influence *Campylobacter* infections, including age (higher incidence in children under the age of 5), the season (more prevalent during summer months), the strain variation (certain strains are less virulent than others), and demographic factors (social economic status), all of which contribute to the morbidity and mortality worldwide (129) . *Campylobacter* is highly infectious and infective doses as low as 500 to 800 CFU have been reported in human patients (66). The organism is transmitted to humans through a variety of sources, such as contact with infected pets or consumption of contaminated milk or water (80). However, the most frequent source of *C. jejuni* infection is contaminated, undercooked poultry meat (41,44,50). In humans, *C. jejuni* colonizes the intestinal epithelium and often causes a mild, watery diarrhea to severe, bloody diarrhea. *Campylobacter* enteritis may occasionally lead to the development of immune-mediated neurological sequelae known as Guillain-Barré syndrome (GBS)(159).

Campylobacter spp. are widespread among livestock and poultry, usually colonizing the intestines without causing clinical diseases, however, they may be associated with important illnesses, including infertility and abortion in ruminants (43,128,133). *Campylobacter*-associated abortions in domestic ruminants are an important management problem in agriculture, and can be a major economic loss to producers (133). *Campylobacter* infection is one of the most prevalent causes of ovine abortion in the United States and worldwide, with an overall abortion rate of 5 to 50% (average, 23.2%) in affected flocks (128,133). In susceptible pregnant ewes, initial oral exposure may be followed by bacteremia with subsequent placentitis, fetal infection, and abortion, which usually occurs in the last trimester of pregnancy (128,133). Effective intervention strategies to mitigate sheep abortion caused by *Campylobacter* have been hindered mainly because of gaps in our understanding of its virulence mechanisms, the lack of an adequate animal model for the disease, and a growing incidence of antibiotic resistance strains or vaccine failures (38).

Despite being a major cause of enteric disease in humans and infectious abortion in animals, significant progress has been made during the last decade in understanding the pathobiology of *C. jejuni*. Specific virulence mechanisms for *Campylobacter* remain poorly understood, in part due to the lack of efficient genetic tools and disease-duplicating animal model systems. A few putative virulence factors have been discovered, including flagella-mediated motility, bacterial adherence to intestinal mucosa, the ability to invade enterocytes, the ability to produce toxins, capsular polysaccharide and the pVir plasmid (7,38). For *C. jejuni* to reach the gastrointestinal tract it must survive the stresses encountered in the stomach and small intestine due to gastric acid and bile salts, respectively. It is known that flagellar motility is required for colonization of the mucosal layer, where the organism can

adhere to the host's intestinal cell surface and produce cytotoxins. In most cases, *C. jejuni* only causes a localized infection in the gut and does not spread systemically, however highly virulent *C. jejuni* strains may be able to penetrate the epithelial barrier and spread to extraintestinal sites, such as the liver, gallbladder, pancreas, uterus, and fetal tissues (135).

Ongoing work at Iowa State University has demonstrated that a single clone of *C. jejuni*, named clone SA (for sheep abortion), has emerged in recent years as the predominant cause of *Campylobacter*-associated sheep abortions and has also been identified to be a cause of bovine and caprine abortions in the United States (30,119,128). The exact mechanisms that this pathogenic clone uses to cause abortions remain largely unknown. Using an established pregnant guinea pig model, we demonstrated the high virulence property of *C. jejuni* clone SA in inducing abortion compared with other *C. jejuni* strains (29,30). Although clone SA is highly abortifacient in sheep and guinea pigs, bacterial factors involved in this process are largely unknown. To begin to understand how clone SA causes abortion, we have recently obtained the complete genome sequence of a clinical isolate of clone SA, IA3902 (GenBank accession no: CP001876.1 and CP001877.1). Although comparative genomics analyses indicated presence of a few genes that are uniquely associated with this clone SA strain, its genome shows overall remarkable similarity to those of non-abortifacient isolates, implicating that it must possess unique and complex virulence attributes allowing it to be a successful invader of the fetoplacental unit.

To study pathogenesis in detail, an efficient animal model is needed to assess invasion of the intestine and the subsequent induction of bacteremia. Although the pregnant guinea pig model is appropriate for investigating *Campylobacter*-associated abortion in sheep, it is cost-prohibitive and presents ethical concerns, as it involves pregnant animals.

On the other hand, mouse models for *Campylobacter* infections have shown that bacteremia is a common phenotype for invasiveness and can be consistently measured with this model system (22,24,113,115,149). Here, we report recent progress in developing a mouse model suitable for examining critical steps of *C. jejuni* pathogenesis and identify the capsule as a key virulence factor of this pathogenic organism's ability to induce bacteremia. The outcomes of this study improve our understanding of pathogenesis of abortion caused by *C. jejuni* clone SA, and aid in the development of means to control this major disease in sheep.

Thesis Organization

This thesis consists of three chapters. The first chapter is a literature review that includes a general overview of *Campylobacter* (mainly *C. jejuni*) and *Campylobacter* abortion, key elements for pathogenesis, and current animal models. The second chapter describes the development of a mouse model for *Campylobacter*-induced bacteremia and the investigations on virulence traits of clone SA. The third chapter is a general conclusion. The references cited throughout the text are located immediately after each chapter.

Literature Review

Taxonomy and biology

Although McFadyean and Stockman isolated the first *Vibrio*-like bacteria from aborted ovine *fetus*es in 1913 (147), it is believed that the first observation of *Campylobacter* occurred in 1886 by Theodor Escherich, who described non-culturable spiral-shaped bacteria (92,147,148). In the following decades, several spiral-shaped organisms were detected

primarily by veterinarians, with these ultimately being designated as *Vibrio* species. In 1927, Smith and Orcutt named a group of bacteria isolated from the feces of cattle with diarrhea as *Vibrio jejuni* (129). Similarly, in 1944, Doyle isolated yet another vibrio from feces of pigs with diarrhea and classified them as *Vibrio coli* (92,129,147).

As a result of their low G + C content in chromosomal DNA, their microaerophilic growth requirements and their nonfermentive metabolism, Sebald and Véron proposed the genus *Campylobacter* in 1963, distinguishing them from the "true" *Vibrio* species. Since then, advances in methods for selective isolation of *Campylobacter* and taxonomic tools such as molecular methods have resulted in many more species being added to the genus, albeit with some misclassifications. The family *Campylobacteraceae* consists of the genera *Campylobacter, Arcobacter, Sulfurospirillum* and *Bacteroides ureolyticus*, all of which occur primarily as commensals in humans and domestic animals (147). The genus *Campylobacter* is currently comprised of 14 phenotypically and genotypically heterogeneous species: *C. jejuni, C. coli, C. lari, C. upsaliensis, C. helveticus, C. fetus, C. hyointestinalis, C. mucosalis, C. concisus, C. curvus, C. showae, C. rectus, C. sputorum* and *C. gracilis*.

Campylobacter species are small (~0.5 µm wide and ~3 µm long), Gram-negative, spiral or curve shaped rods. Most species possess a single, unsheathed polar flagellum that is present at one or both poles, giving the cell rapid motility in viscous environments, such as the gastrointestinal mucosal layer (52). Some species are non-motile (*C. gracilis*) or have multiple flagella (*C. showae*). *Campylobacter* species are microaerophilic, growing best in an atmosphere with low oxygen tension (5% O_2, 10% CO_2 and 85% N_2) (51). The optimum growth temperature for *Campylobacter* lies between 37 and 42°C. Typical biochemical

characteristics include the presence of oxidase activity and hippurate hydrolysis (only *C. jejuni*). *Campylobacter* spp. are fastidious organisms, which do not readily ferment carbohydrates, but are thought to primarily use amino acids as their carbon source (147).

Genomic Characteristics

Since most of the research on *Campylobacter* has been focused on *C. jejuni*, this literature review will mainly focus on *C. jejuni* as an important pathogen. *C. jejuni* has a relatively small circular genome between 1.6 and 1.8 megabases with a rather low G + C content of 30% (52). It is naturally competent, meaning that it can take up DNA from the environment, giving way to more genetic diversity, including antibiotic resistance (159). Since the publication of the complete genomic sequence of *Campylobacter jejuni* NCTC 11168 in February 2000 (114), mounting evidence suggests that *C. jejuni* strains are genomically diverse (42,99,114). Several studies have been aimed at describing this diversity. For example, unique genomic DNA sequences of *Campylobacter jejuni* 81-176 were identified by comparison with *C. jejuni* NCTC 11168 (121). *C. jejuni* 81-176 carries two large plasmids, named pVir and pTet (13,16) while 11168 does not possess either one of the plasmids (15). Recently, we discovered a highly virulent *C. jejuni* clone (clone SA for sheep abortion) that is responsible for the majority of ovine abortions in the United States (128), and determined the complete genome sequence (GenBank accession no: CP001876.1 and CP001877.1) of a clinical isolate of clone SA (IA3902). Comparative genomics with the non-abortifacient NCTC 11168 indicated the presence of a number of genes that are uniquely associated with this strain (Q. Zhang laboratory, unpublished data). These genomic

differences may help explain the observed difference in virulence between clone SA and other strains of *C. jejuni.*

Pathogenesis

As a zoonotic pathogen, *Campylobacter* colonizes the gastrointestinal tracts of a variety of animal hosts, either as a commensal or as a pathogen (80). *C. jejuni* is an enteric organism and is transmitted by the fecal-oral route. To establish an infection, *C. jejuni* must first survive the acidic conditions in the stomach, and then colonize the large intestine (120,131,159). Colonization of the gut mucosa depends on flagellar motility and corkscrew morphology of the organism, and its ability to adhere to the mucosal layer (131,159). Once established, it activates other virulence factors and toxins to cause inflammation and epithelial damage with fluid secretion (152). However, our understanding of these virulence processes in *Campylobacter* pathogenesis is poorly developed compared with other enteric pathogens.

Despite our limited knowledge of the pathogenic mechanisms of this organism, flagella-mediated motility, bacterial adherence to the intestinal mucosa, invasiveness and the ability to produce toxins have been identified as virulence factors allowing *C. jejuni* to colonize the host (7,38,129,146). *Campylobacter* also possesses mechanisms for adaptation to various environmental conditions such as iron limitation, temperature fluctuations, and oxygen rich atmosphere (53,58).

Although various virulence factors have been implicated (15,19,96), the precise mechanism(s) contributing to the pathogenesis of *Campylobacter* spp. have yet to be

elucidated, partly because of the lack of a suitable animal model and partly due to difficulties in genetic manipulation of this organism (146).

a) Flagella

C. jejuni flagella and flagellar motility are vital to many aspects of *C. jejuni* biology, including host colonization, host cell invasion and secretion. Flagella are involved in auto-agglutination (103) and biofilm formation (85,86). The role of motility in the virulence of *C. jejuni* was established by use of isogenic non-flagellated mutants unable to colonize the intestine of experimental animals (38,157). Furthermore, Wassenaar and Blaser (153) demonstrated that aflagellated *C. jejuni* mutants show a significant reduction of internalization by host cells, suggesting the importance of flagella in invasion. Structurally, the flagellar filament consists of flagellin subunits encoded by two tandem genes, *flaA* (major subunit) and *flaB* (minor subunit) (112). A two-component system comprised of the sensor FlgS and the response regulator FlgR is central for the regulation of the *Campylobacter* flagellum (38). Additional regulation involves *fliA*, *rpoN*, and the housekeeping gene *rpoD* for motility, protein secretion and invasion (31,69,70,77,156). Two proteins, FlgP and FlgQ, were shown to be essential for flagellar motility in *C. jejuni*, however, their functional roles remain unclear (137). Homologs of other important flagellar gene regulators like FlhC and FlhD, found in other bacterial species have not been identified in the *C. jejuni* genome (114). In addition to agglutination, attachment and biofilm formation, bacterial flagella also enable chemotaxis, a behavior that plays an important role in both the commensal and pathogenic lifestyle of *C. jejuni* (159). Together, these data demonstrate that flagella and associated

components are crucial factors for the initial interaction of *C. jejuni* with its host, leading to colonization of the intestine.

b) Lipooligosaccharide and capsule

Surface polysaccharides represent the predominant structures on all bacterial cell surfaces, and they are often important in the interactions between pathogens, their hosts and the environment (57,62). *C. jejuni* is polysaccharide-rich, harboring four well-defined carbohydrate biosynthetic loci encoding proteins responsible for biogenesis of the lipooligosaccharide (LOS), capsular polysaccharide (CPS), O-linked flagellar sugars and N-linked protein glycans. The LOS and CPS produced by different strains of *C. jejuni* are structurally complex and highly variable. Many genes in these hypervariable regions are also subject to phase variation, further producing genetic and phenotypic diversity and confounding immune system responses to *C. jejuni* infection (108).

As in other enteric pathogens, *C. jejuni* expresses a phase-variable outer LOS core capable of mimicking human antigens. *C. jejuni* is one of a few bacteria that are capable of the endogenous synthesis of sialic acid for incorporation into these ganglioside-like LOS cores (65). The LOS molecule is comprised of two components: a lipid A anchor and an oligosaccharide consisting of a conserved inner core and a variable outer core, therefore it is referred to as LOS, because it lacks the O-antigen found in lipopolysaccharides (59,71,88). LOS is an important factor in *Campylobacter* pathogenesis. Currently, the best-characterized contribution of the *C. jejuni* LOS to human disease is its relationship to the debilitating neuropathy Guillain-Barre syndrome, GBS (106,142), in which the LOS structures mimic the human gangliosides on peripheral nerves. Depending on phase variation of the *cgtA* gene,

the 81-176 LOS can mimic human GM2, GM3, CD1b and GD2 gangliosides (57). Numerous studies have shown that various LOS mutants are surprisingly sensitive to certain antimicrobial substances (79,87,100,101). Despite its contribution to chronic sequelae such as GBS, no direct pathogenic role for the LOS has been definitively identified.

The capsular polysaccharide (CPS) is the outermost structure on the bacterial cell, and possibly plays a key role in the interaction between the pathogen, host and environment (142). Generally, CPS is thought to be important for bacterial survival and persistence in the environment and often contributes to pathogenesis (124). CPS of *C. jejuni* remained elusive until the sequencing of NCTC 11168 revealed a set of genes that are homologous to the type-II and type-III capsule transport genes of the *Enterobacteriaceae* (114). For *C. jejuni*, the CPS is considered to be an important virulence factor based on its involvement in epithelial cell adherence, invasion and serum resistance (14,71). Encapsulated bacteria thwart the antibacterial effect of complement by preventing deposition of the host membrane attack complex on bacterial membranes (104). Mutation in the CPS transporter gene, *kpsM*, results in the loss of the capsule, and was found to abolish colonization of chickens (14,84),while mutants of systemic strains (*C. jejuni* 84-19 and 84-25) were >1,000-fold more sensitive to complement-mediated killing compared to their respective wild-type strains (90). A *C. jejuni* mutant for the *kpsE* gene, which is unable to express any capsular polysaccharide, was not hampered in its ability to colonize the chicken intestinal tract, but the number of bacteria recovered from the cecum and colon were lowered compared to the control (12). The CPS is phase variable in expression, presumably due to slip-strand mispairing occurring in genes essential for CPS synthesis, a mechanism of variation that is common in *C. jejuni* (63,114,142). The high degree of variability of CPS genes is consistent with CPS being the

major serodeterminant of the Penner or heat-stable (HS) serotyping scheme (88). However, it is not known whether differences in sugar composition of the capsule among strains affect virulence or whether the phase-variable modifications have a role in pathogenesis or interactions with bacteriophages (36).

c) Cytolethal distending toxin

A variety of toxic activities have been attributed to *C. jejuni*. However, cytolethal distending toxin (CDT) is the only verified *Campylobacter* toxin identified. It is produced by a number of *Campylobacter* spp. including: *C. jejuni*, *C. lari*, *C. coli*, *C. fetus* and *C. upsaliensis* (38,83). It has been described as an important virulence factor of this pathogen (6). Significant progress has been made in the past several years that have led to a rapid advancement of our understanding of the role of CDT in pathogenesis (14,72,88,89). CDT holotoxin is composed of three subunits encoded by the *cdtA*, *cdtB* and *cdtC* genes (118), and causes eukaryotic cells to arrest in the G2/M phase of the cell cycle preventing them from entering mitosis and consequently leading to cell death (54,118,162). It is now clear that CdtB is the active moiety of the Cdt ABC complex. It appears that CdtA and CdtC interact with CdtB to form a tripartite CDT holotoxin necessary for the delivery of the enzymatically active subunit, CdtB (97). After that, the CdtB active subunit, which has DNaseI-like activity, induces host DNA damage by breaking its double strand (54). Wild-type *C. jejuni* strain NCTC 11168 produces more toxin and is highly invasive in SCID mouse tissue compared to isogenic *cdtB* mutants, which are unable to produce noticeable amounts of toxin (38); similarly, isogenic *cdtB* mutants of *C. jejuni* strain 81-176 produce remarkably reduced levels of CDT (123). It is not clear yet what role CDT plays in infection *in vivo*. However, it

may contribute to the invasiveness and modulation of the immune response rather than directly to inducing diarrhea (123).

d) Adhesion and invasion

Many studies have shown that *C. jejuni* requires adhesion and binding factors to colonize hosts. These experiments have led to the identification of some putative adhesins or binding factors of *C. jejuni* including fibronenctin-binding outer membrane protein CadF (94), the periplasmic binding protein PEB1 (116), the autotransporter CapA and the surface-exposed lipoprotein JlpA (81). CadF is an outer membrane protein (OMP) expressed in all C. *jejuni* and *C. coli* strains and mediates cell adhesion by binding to the cell matrix protein, fibronectin (38). CadF is required for maximal binding and invasion by *C. jejuni in vitro*, and *cadF* mutants are greatly reduced in chick colonization compared with the wild-type (105,163). PEB1 is an adhesin, and also functions as the periplasmic-binding protein component of amino acid ABC transporters (116). PEB1 binds to both aspartate and glutamate, and *peb1* mutants cannot grow if these amino acids are the major carbon source (98). Thus, the PEB1 transport system appears to have an important role in utilization of aspartate and glutamate, however, its function *in vivo* as an adhesin in *C. jejuni* is unknown. JlpA is a surface-exposed lipoprotein that is crucial for the binding to host epithelial cells (81). One study indicated that JlpA interacts with Hsp90α, triggering signaling pathways that activate NF-κB and p38 MAP kinase, both of which are components involved in host proinflammatory responses to infections (82). CapA is another autotransporter lipoprotein that has been identified to adhere to human and chicken epithelial cells (8).

Cellular invasion is an important pathogenic mechanism of *C. jejuni*. The invasion of epithelial cell *in vivo* results in cellular damage and function loss, which leads to stimulation of host inflammatory responses and diarrhea (153). Furthermore, it has been observed that diarrheal disease is correlated with the invasiveness of *C. jejuni* (127). Similarly, numerous studies have established that early mucosal damage is a result of invasion of *C. jejuni* in the colonic epithelial cells (49,74,155), ultimately leading to the conclusion that the invasive capability of this organism is an important pathogenic factor. However, different strains of *C. jejuni* vary in their ability to invade.

Many invasive pathogens subvert host cytoskeletal structures as part of the pathogenic process. The highly invasive *C. jejuni* 81-176 demonstrated microtubule-dependent invasion, and appears to rely on microtubule motors for uptake and intracellular motility (25,73). However, in rabbit intestinal models, *C. jejuni* selectively associates with M cells (48,151), suggesting that this cell type may be an important port of entry. The mechanism starts with the efficient migration of the pathogen (neither strain- nor cell type-specific) into the subcellular space, followed by a rapid and highly efficient bacterial invasion at the basal cell side (145). Regardless, most *C. jejuni* strains exhibit microfilament-dependent or microfilament/microtubule-dependent invasiveness (20).

Animal Models of *Campylobacter jejuni* Infections

Despite its obvious global importance, relatively little is known regarding mechanisms of *C. jejuni* pathogenesis and host responses to infection. Many studies have sought to establish animal models for study of *C. jejuni* colonization and gastroenteritis, but the lack of high-resolution, facile and reproducible models for studying infection *in vivo* has

clearly hampered progress in this area (33). Newborn pigs, weanling ferrets, gnotobiotic canine pups, and nonhuman primates have been inoculated experimentally with *C. jejuni* by various routes to mimic the course of infection, however, these models suffered from high costs, difficulties in animal handling and availability, lack of reproducibility, or inadequate biological characterization (11,17,18,33,126,158,160). Although the chick model allows to us investigate colonization and transmission, disease-defining diarrhea is absent, since *C. jejuni* appears to be a commensal organism in this animal. Regardless, significant progress has been made with these models to understand pathogenic mechanisms of *C. jejuni*.

Reliable murine models of *C. jejuni* infection overcome some of these limitations, and offer the advantage of an immune system that is well characterized and has been used extensively. Other advantages are the animals' small size, wide availability, inexpensive housing costs, defined microbial flora, and amenability to manipulation. A number of investigators have shown that oral dosing of *C. jejuni* in mice of different strains, both inbred and outbred, results in intestinal colonization and in some cases bacteremia, but the organism does not usually cause clinical diarrhea (2,22,138). The mouse model also highlighted the essential nature of the flagella in colonization of the intestinal mucosa (2,40,111), as well as the importance of *peb1A* in *C. jejuni* adhesion (117).

Furthermore, intraperitoneal (IP) inoculation of pregnant guinea pigs has been used as a method to evaluate the pathogenicity of *Campylobacter* spp. (141,143) and to assess the efficacy of vaccines (26-28). Their small size, ease of housing, and relatively short duration of gestation make guinea pigs a desirable animal for use in studying *Campylobacter*-induced abortion, particularly when compared with the use of sheep or goats, which are the species most commonly affected in nature. IP inoculation is not the best method for evaluating

Campylobacter pathogenesis of septic abortions, because it avoids the critical steps required for abortion (intestinal colonization and bacterial invasion). A recent study (30), reported that oral inoculation of a highly virulent *C. jejuni* strain (clone SA) was effective in inducing abortion in pregnant guinea pigs.

Clinical aspects of *Campylobacter* disease

a) Campylobacteriosis in humans

Campylobacter jejuni infection is one of the most commonly identified bacterial causes of acute gastroenteritis worldwide (5). The clinical presentation of patients with *Campylobacter* infection differs between developing and industrialized countries. In developing countries, *C. jejuni* infection is milder than in developed countries. Infection can be asymptomatic or there may be mild non-inflammatory diarrhea, predominantly affecting young children (37). In the developed world, *Campylobacter*iosis manifests with abdominal pain, diarrhea and fever, which is typically self-limiting (159). Affected individuals experience varying degrees of diarrhea, which may range from a few loose stools to profuse watery diarrhea causing dehydration or less often bloody diarrhea with mucus. The incubation period that precedes the development of acute diarrhea is 2-5 days, and although the disease is typically resolved in one week, symptoms can last up to two weeks. Although a large proportion of the patients feel nauseous, only about 15% of patients vomit (131). In 30% of patients, the disease does not start with diarrhea but with a prodrome of influenza virus-like symptoms such as fever, headache, dizziness, and myalgia, indicating that there is some systemic, probably immune-mediated, effect of local infection (78,154). Variations in

bacterial virulence or host immune response each may play a role in these different phenotypic expressions of the disease (37,91).

While *Campylobacter* enteritis is usually self-limiting, some individuals develop sequelae after the acute phase. Approximately 1 in 1,000 infected individuals develops Guillain-Barré syndrome (GBS), a serious autoimmune-mediated neurological disorder that can cause symptoms ranging from weakness of extremities to complete paralysis and respiratory insufficiency (78). Molecular mimicry between peripheral nerve glycolipids or myelin proteins and structures on the lipopolysaccharides of some *Campylobacter* strains likely plays a role in the pathogenesis of *Campylobacter*-induced GBS (5,161).

b) Campylobacter in Sheep

Campylobacter infection is one of the most prevalent causes of ovine abortion in the United States and worldwide (128). *Campylobacter* species can be carried in the intestines and gall bladder of healthy sheep without causing clinical diseases (3,102,128,139). However, highly virulent *C. jejuni* strains can invade into circulation and cause systemic infections (135). In susceptible pregnant ewes, initial exposure may be followed by intestinal invasion and bacteremia with subsequent placentitis, fetal infection, and abortion, which usually occurs in the last trimester of pregnancy (35,130,133,135). During the initial period of infection, ewes usually do not show any clinical signs of disease; however, occasionally ewes die due to uterine sepsis and septicemia if the fetus dies and is retained in utero (128,133). If the onset of infection is delayed closer to term, lambs are born weak and typically do not survive. Gross lesions in aborted ewes include thickened uterine walls with edema, swollen caruncles covered with exudate and placentas with mottled swollen

cotyledons (68). Aborted fetuses can be mildly to severely autolyzed and commonly have serosanguinous fluid in the abdomen and thorax and less often, focal liver necrosis (68,93,128,133). Histologically, the aborted placentas often have the signs of acute suppurative placentitis with congestion and necrosis of cotyledons and trophoblasts can be distended with intracytoplasmic organisms that can be stained with Giemsa stain (68,93). A mild to moderate suppurative fetal bronchopneumonia typically is identified. Less consistently, the livers of aborted fetuses have multifocal areas of necrosis surrounded by a thin to moderate infiltrate of neutrophils.

c) Bacteremia

In immunocompetent individuals, disease is usually restricted to the intestine, although bacteremia and systemic infection have been observed. Bacteremia resulting from *Campylobacter*iosis is uncommon and transient in immunocompetent people (134). Bacteremia is more common in patients ≥65 years of age with a *C. jejuni* infection. Highly virulent *C. jejuni* strains may be able to penetrate the epithelium barrier and spread to extraintestinal sites, such as liver, gallbladder, pancreas, uterus and fetal tissues (135). Once the intestines have been colonized, abortifacient *Campylobacter* spp. must breach the intestinal epithelium and induce bacteremia. Bacteremia attributable to *C. jejuni* has been described (23,95,150), and the results of one study (134) indicated that certain strains of *C. jejuni* have enhanced ability to induce bacteremia. Therefore, bacteremia is an essential factor for systemic infection, enabling the organism to reach the fetoplacental unit, where multiplication results in placentitis and eventually leads to abortion (35,130,133,135). Less is known about the pathogenesis of *C. jejuni* infections that gain access to the vascular space

and cause systemic infection. When a systemic *C. jejuni* infection occurs in an apparently healthy individual, the recovered isolate is likely to have features promoting persistence in the bloodstream that distinguish it from diarrheal *C. jejuni* strains (23).

Epidemiology

C. jejuni resides in the intestinal tracts of many wild and domestic animals as a commensal organism. The avian species are the most common hosts for *Campylobacter* probably because of their higher body temperature (129,132). *Campylobacter* appears to permanently colonize the gastrointestinal tract of birds with few noticeable ill effects and only occasionally is diarrhea observed with *Campylobacter* infection in young animals (110). Shedding of *Campylobacters* by birds (very high *Campylobacter* carriage rates have been reported among geese and ducks (10,122), among others) causes contamination of waterways, and as *Campylobacters* can survive in water for weeks, open waters may then act as a source of infection for domestic animals (37).

A large number of published studies on the incidence of *Campylobacter* spp. in food animals confirm that these human pathogens are also commonly found in many types of food animals other than chicken, e.g., cattle, pigs, dairy cows, turkeys, duck or lamb (75). The digestive tract of healthy cattle has been demonstrated to be a significant reservoir for a number of *Campylobacter* spp., with prevalence in cattle ranging from 0-80 % (9), while the prevalence in sheep is about 20% (164). In addition, high prevalence in pigs has been reported (109).

Recent studies performed by our group (128) made the observations that a highly pathogenic *C. jejuni* clone (clone SA) has become a persistently predominant cause of

Campylobacter-associated ovine abortions in the United States, suggesting that the clone is ecologically well adapted and pathologically hypervirulent in ruminants (107). Also, clone SA was detected in healthy sheep and cattle, and abortion cases in cows and goats, indicating that the clone is present in the ruminants as both commensal and a pathogen. The presence of *C. jejuni* clone SA in the bile of healthy sheep suggests that it may colonize the gall bladder, which would facilitate the persistence of clone SA in sheep.

The majority of *Campylobacter* infections in humans are sporadic and predominantly associated with poor handling of raw chicken or consumption of undercooked chicken (41,44,50). Other risk factors include contact with house pets, exposure to farm animals, and the consumption of raw milk, untreated water, and undercooked beef, pork, and shellfish (21,44). Outbreaks due to *Campylobacter* are most commonly associated with consumption of raw milk, contaminated surface water, and chicken meat (21,60,61). Despite the prominent role of poultry in the transmission of campylobacteriosis, recent molecular epidemiological studies indicate that ruminants also contribute significantly to both sporadic cases and the outbreak of human infections via contaminated milk, water and produce (34,39,67,107).

Treatment and resistance

As *C. jejuni* infection is usually self-limited, in most cases there is no rationale for antimicrobial treatment. Supportive measures, such as fluid and electrolyte replacement, are the principal therapies for most patients with campylobacteriosis. Antibiotic therapy, however, should be considered for individuals who have high fever, bloody diarrhea, prolonged infection, pregnancy, HIV or are immunocompromised. Antibiotics, generally

macrolides, tetracycline and fluoroquinolones, are reserved for more severe cases. However, the increasing resistance to fluoroquinolones, tetracycline and erythromycin of *C. coli* and *C. jejuni* strains, might compromise the efficacy of this treatment (1,4,47,55). Currently, erythromycin is the drug of choice for most *Campylobacter* infections, because of its low cost, ease of administration, lack of serious toxicity, and narrow spectrum of activity (5).

Antibiotic resistance among *Campylobacter* has become a serious problem worldwide. Fluoroquinolones have become less effective as resistance has increased dramatically (47). Since antibiotics have been indiscriminately used in animal production for decades in order to control, prevent and treat infections, and enhance animal growth (45,76,125), there is strong evidence that supports the hypothesis that the unregulated use of antimicrobial agents in food animal production has led to the emergence and spread of antibiotic resistance among *Campylobacter* spp. (129). Smith (136) demonstrated a significant increase in quinolone resistance among human *Campylobacter* isolates between 1996 and 1998 that was temporally associated with the licensure of fluoroquinolones for use in poultry in the United States.

The tetracycline class of antibiotics (tetracycline, oxytetracycline, and chlortetracycline) is commonly used in the prevention and control of abortion storms associated with *Campylobacter* spp. in the United States (56); however, speculation from clinical veterinary practitioners has suggested an increasing ineffectiveness of these drugs in treating *Campylobacter*-associated abortions (128). A recent study performed by Sahin et al. (128) determined the antimicrobial susceptibility patterns of *C. jejuni* isolates from sheep abortion to the drugs that are commonly used in food animal production; strikingly, the results demonstrated that 100% of the *C. jejuni* isolates associated with sheep abortions

across the U.S. were resistant to oxytetracycline, suggesting that this class of antibiotics is no longer effective in treating *Campylobacter*-induced abortion.

Prevention

As the primary source of *Campylobacter* infection in humans is foodstuffs, particularly poultry, prevention should be aimed at reducing the infection level among poultry houses. Installing hygienic barriers between the external and internal environments, such as controlling the entry of farm personnel, strict hygienic routines such as washing and sanitizing of hands, changing boots and coveralls before entering, have been shown to be effective, but these control measures are easily and often breached (129). Reducing the level of *Campylobacter* during slaughter and preventing cross-contamination of carcasses is an ideal way to control human infection. Public education programs emphasizing good hygiene and food preparation techniques are also important in the prevention of *Campylobacter* infection.

The use of antibiotics in food animal rearing has been hotly debated since speculations have suggested that this has given rise to antibiotic-resistant strains, severely limiting the efficacy of antibiotics in treating human disease (46,47,64,136,144). However, the use of pre- and pro-biotics (i.e., complex polysaccharides and strains of lactic acid bacteria), competitive exclusion, application of bacteriocin-producing bacteria, and bacteriophages have shown some promise (129,140).

Vaccination against *Campylobacter* has been used to control sheep abortion worldwide. Currently, there are at least two vaccines available in the United States used for protection against sheep abortions caused by *Campylobacter*; however their efficacy varies

widely. It is likely that the strains incorporated in these formulations were those that were the most common species/serotypes at the time the vaccine was created. Thus, new vaccines are necessary to account for the increasing role of *C. jejuni* in sheep abortions.

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CHAPTER 2. SYSTEMIC INFECTION INDUCED BY *CAMPYLOBACTER JEJUNI*: DEVELOPMENT OF A MOUSE MODEL AND ELUCIDATION OF MOLECULAR MECHANISMS

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Abstract

Campylobacter jejuni clone SA has emerged as the predominant cause of *Campylobacter*-associated ovine abortion in the U.S., and this clone is highly pathogenic in pregnant sheep and guinea pigs. To induce abortion, orally ingested *Campylobacter* must be able to translocate across the intestinal epithelium and spread systemically. To understand the pathogenic mechanisms and immune protection of *C. jejuni*-induced abortion, it is necessary to develop a cost-effective animal model to evaluate systemic infection induced by this pathogenic clone. In this study, two different breeds of female mice (BALB/c and CD-1) were orally inoculated with *C. jejuni* IA3902, a clinical clone SA isolate whose complete genome sequence has been determined, to evaluate the induction of bacteremia and hepatic infection. Our results revealed that CD-1 mice were more susceptible than BALB/c mice to infection by IA3902. In CD-1 mice, *C. jejuni* IA3902 induced bacteremia and hepatic infection within 1 hour after oral inoculation, and bacteremia peaked at 8 and/or 12 hours after inoculation. Compared with IA3902, the magnitude and duration of bacteremia and hepatic infection induced by *C. jejuni* strains NCTC 11168 and 81-176 were significantly less prominent, indicating that IA3902 is more virulent than the other strains tested with

regard to systemic spread. Mutagenesis in IA3902 showed that the loss of the capsule (Δ*kpsS*) completely prevented the organism from causing bacteremia and hepatic infection, while the loss of pVir plasmid did not affect systemic spread. These findings indicate that the CD-1 mouse model is suitable for examining critical steps of *Campylobacter* pathogenesis and identify the capsule as a key virulence factor of this pathogenic organism to induce bacteremia.

Introduction

Campylobacter species have been recognized as one of the most prominent causes of ovine abortion in the United States and worldwide, with an overall abortion rate of 5-50% in affected flocks (55). A recent national study, NAHMS Sheep 2001, conducted by USDA/APHIS/Veterinary Services in collaboration with the American Sheep Industry Association, revealed that *Campylobacter* species ranked first among all infectious causes of abortion within the last 3 years of the study, with 53.7% of the reported cases confirmed by a veterinarian or diagnostic laboratory (62).

Historically, *C. fetus* subsp. *fetus* accounted for the majority of the *Campylobacter* spp. associated with ovine abortion, however, we and others recently discovered a remarkable shift in the etiology of the disease (22,53). Specifically, a highly virulent *C. jejuni* clone (clone SA for **s**heep **a**bortion) has replaced *C. fetus* as the predominant cause of ovine abortion outbreaks in the U.S. Considering the high genetic diversity in *Campylobacter* species and the strains responsible for sheep abortion in the U.S. prior to the predominance of the SA clone (1,44,57), this finding was quite surprising and indicates that clone SA has evolved to possess novel virulence traits and agricultural practices in sheep

production have favored the dominance of this hypervirulent clone. The high virulence of clone SA in causing abortion was confirmed using a pregnant guinea pig model, in which the distinct abortifacient ability of the clone was shown compared with other *C. jejuni* strains (41).

Campylobacter species usually live as commensals in the intestines and gall bladder of healthy sheep without causing clinical diseases (1,44,57). However, highly virulent *C. jejuni* strains, such as the SA clone can cause systemic infections. In susceptible pregnant ewes, initial ingestion of *C. jejuni* is believed to be followed by intestinal invasion and bacteremia with subsequent placentitis, fetal infection and abortion (55). The ability of the organism to induce bacteremia is one of the key elements in the abortion process. To facilitate bacteremia, *C. jejuni* must first invade the mucosal tissue of the intestinal tract, consequently surpassing the host immune responses in the blood long enough for bacteremia to spread systemically. Clone SA is highly abortifacient in both sheep and the pregnant guinea pig model, and therefore, it must possess distinctive virulence characteristics enabling it to invade the host intestine and bloodstream.

Despite these advancements in understanding *Campylobacter* pathogenesis and its obvious global importance, little is known about the molecular mechanisms of *Campylobacter*-associated abortion. The lack of high-resolution, tractable and reproducible models for studying infection *in vivo* has clearly hampered progress (18). An optimal animal model to study *Campylobacter* pathogenesis should comprise several features (70). Symptoms similar to those observed in abortion cases are required after oral administration of bacteria to animals. Sheep are generally silent carriers and do not exhibit any symptoms of clinical disease. Although the pregnant guinea pig model resembles *Campylobacter*-

induced abortion in sheep (17), it is cost prohibitive (>$300 per animal), time consuming and cumbersome, and is not feasible for large-scale studies involving multiple experiments. Murine models of *C. jejuni* infection overcome some of these limitations, and therefore, are useful for assessing systemic infection. These mouse models have been used to study colonization and/or virulence mechanisms and host responses (30,36,51,61,64), to screen natural isolates or laboratory strains carrying spontaneous or targeted mutations that are thought to affect colonization and/or virulence (11,28,40,42,50,63). Furthermore, bacteremia was a very common and consistent finding in several studies (8,12,45,48), suggesting the suitability of the mouse model for studying *Campylobacter* pathogenesis. We hypothesize that mice are susceptible to the highly pathogenic clone SA and can provide a suitable model for investigation of systemic dissemination of pathogenic *Campylobacter* in infected animals.

Surface polysaccharides represent the predominant structures on bacterial cell surfaces, and they are often important in the interactions between pathogens, their hosts and the environment (24). *C. jejuni* is now known to produce capsular polysaccharide (CPS) (19,34,46). CPS is one of the few known virulence factors of *C. jejuni* that are required for diarrheal disease in ferrets, chicken colonization, the adherence and invasion of human epithelial cells, serum resistance and surface charge (5,31). Karlyshev et al. (33) have shown that site-specific insertional mutagenesis of polysaccharide transporter genes *kpsM*, *kpsS* or *kpsC* in several strains resulted in the loss of a high-molecular-weight glycan, providing isogenic mutant strains for evaluation the role of CPS in pathogenesis. Despite these studies, the role of CPS in *Campylobacter* bacteremia and systemic infection has not been evaluated.

The pVir plasmid has been recognized as a potential virulence factor in *C. jejuni*, because strains carrying this plasmid had an increased ability to invade INT 407 cells *in vitro* (4-6,69). In addition, previous studies by others reported that pVir contributed to *Campylobacter* virulence in a ferret diarrheal disease model and was associated with bloody diarrhea in *Campylobacter jejuni* enteritis (4,60). Although pVir is not exclusively associated with clone SA isolates, there is a possibility that pVir interacts with chromosomally encoded factors in clone SA in inducing sheep abortion. We cured pVir (Q. Zhang laboratory, unpublished data) and reintroduced the plasmid back into the cured background to assess if the plasmid as a whole contributes to bacteremia in the mouse model, after determining that it is not required for abortion in the pregnant guinea pig model (Q. Zhang laboratory, unpublished data).

In this study, we demonstrate that oral dosing of *C. jejuni* IA3902 (a clone SA isolate) into CD-1 mice leads to efficient establishment and reproducible bacteremia and systemic infection. Since IA3902 is able to successfully induce bacteremia in our murine model it should prove sensitive enough to identify mechanisms necessary for producing bacteremia. Here we report that the loss of capsular expression in IA3902 hinders the ability of the isogenic mutant to produce bacteremia and/or systemic infection. This work establishes a model system that can examine and identify molecular and genetic factors involved in *Campylobacter*-induced bacteremia.

Materials and Methods

Bacterial strains and growth conditions. *C. jejuni* strain IA3902 is a clinical isolate originally isolated from an ovine abortion case described previously (53), and was the wild-

type strain used in this study. *C. jejuni* NCTC 11168 and 81-176 are both human isolates, non-abortifacient, sequenced and used as controls to assess the bacteremic properties of IA3902. All *C. jejuni* strains were grown on Mueller-Hinton (MH) agar under microaerobic (5% oxygen, 10% carbon dioxide, and 85% nitrogen) conditions at 42°C for up to 48 h. As needed for specific experiments, the culture medium was supplemented with *Campylobacter* selective (polymyxin B, rifampicin, trimethoprim, and cycloheximide) and supplemental (sodium pyruvate, sodium metabisulfite, and ferrous sulfate) growth media (Oxoid, Cambridge, England), kanamycin (50 µg/mL) or chloramphenicol (20 µg/mL). *C. jejuni* strains used in this study are listed in Table 1.

Mice. Wild-type BALB/c (inbred) and CD-1 (outbred) female mice 8 to 10 weeks old were purchased from Charles River Laboratories (Wilmington, MA) and maintained by the Division of Laboratory Animal Resources at the College of Veterinary Medicine at Iowa State University. The mice were housed for a minimum of two days before being used for experiments. Following oral inoculation, mice were housed in groups of four or five in sterile polycarbonate microisolator cages with autoclaved bedding and provided with sterilized water and food *ad libitum*.

Oral Challenge. *C. jejuni* used for inoculations was recovered from freezer stocks (-80°C, 20% glycerol), plated on selective medium and incubated for 48 h as described above. Bacterial cultures obtained from these plates were sub-passaged under the same conditions for 18 h. Fresh cultures were harvested and suspended in MH broth, diluted to the desired concentration based on optical density, and subsequently confirmed by viable counts. Each

mouse received 100 µl bacterial culture (approximately 10^8 CFU) via oral gavage using a curved, ball-tipped, 18-gauge, 2-inch needle, under light sedation with 2% isoflurane as previously described (12).

Quantitation of systemic infection. Post inoculation 1, 8, 12, 24 and 48 h mice were anesthetized/euthanized via intraperitoneal (IP) injection of a ketamine/xylazine mixture. Fecal swabs were taken before challenge, and a non-inoculated control group (n=5) was sacrificed at the final time point (48 h) to confirm all mice were *Campylobacter*-free and that no cross contamination occurred. Samples harvested for *Campylobacter* culture included cardiac blood and liver. Cardiac blood was collected by use of a sterile tuberculin syringe with a 22-gauge needle and placed in blood collection tubes (0.5 mL *Greiner Vacuette MiniCollect* K3 EDTA, Fisher Scientific). Within a couple of hours of sample collection, 250 µL of undiluted blood and appropriate serial dilutions thereof were plated on selective medium and incubated for 48 h. Liver tissues were placed in a separate sterile plastic bag (Whirl-Pak bags, Nasco, Fort Atkinson, WI), weighed, homogenized in sterile MH broth, serially diluted, plated on selective medium and incubated for 48 h. *C. jejuni* recovery was expressed as Log_{10} CFU/ml or g of blood or liver. Recovered isolates were subjected to PCR confirmation to assure that the source of infection was the oral inoculation but not environmental or cross contamination.

Construction of a CPS mutant. Construction of the CPS mutant was performed according to the previously reported method (29). Briefly, a 1693bp region harboring *kpsS* (CJSA 1344) was PCR-amplified with kpsS_F and kpsS_R primers (Table 2) and cloned into the

commercial vector pGEM-T (Promega, Madison, WI) to yield pGEM-T::*kpsS*. The resulting amplicon and the pMW10 (67) plasmid, carrying *aphA-3* gene, were digested with *SwaI* and ligated. The suicide vector was delivered to *C. jejuni* IA3902 via natural transformation, and mutants were selected on MH agar plates containing kanamycin (50µg/mL). Δ*kpsS* mutants were confirmed by PCR analysis and Alcian Blue staining.

Alcian Blue staining for CPS. Preparation of CPS samples and Alcian Blue staining were performed as previously described (35). Wild-type and mutant *C. jejuni* cells were harvested following 24hr growth on MH agar, weighed and solubilized in 300 µL of lysis buffer (2% sodium dodecyl sulfate, 4% 2-mercaptoethanol, 10% glycerol, 1 M Tris-HCL, pH 6.8 and 10mg bromophenol blue) for 10 min at 100°C. 20 µL aliquots were taken and mixed with proteinase K (Sigma, St. Louis, MO) to a final concentration of 1 µg/µl and incubated at 50°C for 1 h and fractionated by SDS-PAGE. CPS was visualized with Alcian Blue staining (0.1% Alcian Blue dye, 40% ethanol, 5% acetic acid).

Complementation of the *kpsS* mutant *in trans*. Construction of the complementing plasmid for the *kpsS* mutant was based on a previously published study (25). The entire *kpsS* gene was amplified from strain IA3902 by PCR using primers ST101_F and ST101_R (Table 2). The PCR product was digested with *SacI* and *SacII* and cloned into the plasmid construct pRY112-pABC (39,68) to generate pRY112-*kpsS*, in which the *kpsS* gene was fused to the promoter of *cmeABC*. The constructed plasmid was confirmed by PCR. For complementation, the shuttle plasmid pRY112-*kpsS* was introduced into the *ΔkpsS* mutant by conjugation. The complemented strain was isolated on MH agar containing

chloramphenicol (20µg/mL), and named *ΔkpsS*-comp. PCR analysis and Alcian Blue staining were performed to confirm complementation.

Statistical analysis. Systemic infection results are expressed as the mean with error bars denoting the standard error of the mean (SEM). Statistical analysis of systemic infection results was performed with the Student *t* test. *P* values of less than 0.05 were considered statistically significant.

Results

Effect of mouse strain on systemic infection by *C. jejuni*. Two mouse strains were challenged orally with 10^8 CFU/mL of *C. jejuni* IA3902. The outcome was measured at the ability of IA3902 to cause bacteremia and liver infection following 1, 8, 12 and 24 h post inoculation in BALB/c (inbred) and CD-1 (outbred) mice (Figure 1). Susceptibility was noted in both strains, but CD-1 mice were significantly more susceptible ($p < 0.05$). Therefore, CD-1 mice were selected for subsequent experiments.

Differences of *Campylobacter jejuni* strains in producing bacteremia in CD-1 mice. To assess the ability of clone SA to induce bacteremia, separate groups of mice (n=5) were orally challenged with 10^8 CFU *C. jejuni* IA3902, NCTC 11168 and 81-176, respectively. At 1, 8, 12, 24 and 48 h after oral inoculation, cardiac blood and liver tissues were cultured for *C. jejuni*. The ability to cause bacteremia and liver infection were used as measures of virulence. Pathogenic ability varied as a function of strain and time (Figure 2); IA3902 was the most virulent ($p < 0.05$) with peak infection between 8 and 12 h post-infection (p.i.), and

NCTC 11168 was the least virulent with no observation of bacteremia or liver infection. Strain 81-176 caused only transient infection.

The infected mice did not appear ill or show any clinical symptoms of infection. Animals challenged with IA3902 were bacteremic within 1 h after dosing. The bacterial burden persisted through 48 h at which time the experiment was terminated. Since the liver plays a potential role in clearing *C. jejuni* from the bloodstream, homogenized liver tissues were also cultured. Quantitative cultures of IA3902 in the bloodstreams and the livers of the orally infected mice did not show any significant differences. These results indicate that clone SA possesses a remarkably increased ability to induce bacteremia and liver infection in mice.

Construction and confirmation of CPS mutants of *Campylobacter jejuni* IA3902. To determine the role of *C. jejuni* IA3902 genes involved in inducing bacteremia, we selected the *kpsS* as a target gene to disrupt CPS. The *kpsS* gene encodes an ABC transporter involved in capsule transport (33). Many studies have been conducted investigating the role of *C. jejuni* CPS and its pathogenic properties. While such studies have been useful for defining the general functions of the CPS, we decided to take advantage of the mutants in hand to embark on a comparative analysis of a mutated CPS and its specific aspects of IA3902 pathogenesis. Thus, we conducted an insertional/deletion mutagenesis in *kpsS* of IA3902 in order to define the role of CPS in bacteremia using the mouse model developed. In each mutant strain, a portion of the sequence was replaced with aphA-3 (encoding kanamycin resistance) by double-crossover homologous recombination. Loss of the CPS in the mutant was confirmed by Alcian Blue staining (Figure 3). The CPS complemented strain

(*ΔkpsS*-comp) restored production of normal CPS (Figure 3). The growth rate of the CPS mutant in MH broth was comparable to that of the wild-type (data not shown). Both *ΔkpsS* and *ΔkpsS*-comp grew comparably on selective and nonselective agar plates to that of the wild-type (Table 4), and each exhibited wild-type motility (Table 3).

Mouse model identifies a role for CPS *in vivo*. We hypothesized that the loss of the CPS would make the *ΔkpsS* mutant less virulent than the wild-type, and unable to induce bacteremia and cause subsequent systemic infection. To explore this, we tested the *ΔkpsS* mutant in the mouse model developed in this study. Three groups of CD-1 mice (n=8/group) were orally challenged with *ΔkpsS* mutant, *ΔkpsS*-comp and wild-type IA3902 strains. At 1, 8 and 12 h post inoculation (p.i.), cardiac blood and liver tissues were collected for CFU counts. The *ΔkpsS* mutant exhibited a striking and statistically significant ($p < 0.05$) bacteremic defect evident from 1 h p.i. through 12 h p.i. (Figure 3A and C). The complemented strain caused levels of bacteremia and liver infection similar in frequency and intensity to the wild-type strain.

pVir plasmid as a whole does not contribute to bacteremia. The pVir plasmid is present in some *C. jejuni* strains and carries a putative type IV secretion system, as well as other genes of unknown function. Recently we cured pVir from the SA clone to determine if the plasmid itself contributes to *Campylobacter* abortion in the guinea pig model (Q. Zhang laboratory, unpublished data). To further define the role of pVir, we decided to investigate if the plasmid as a whole contributes to bacteremia in the mouse model. Although pVir-cured strain had significantly less level of bacteremia and liver infection at 8 h p.i., it showed

comparable levels of systemic infection to that of the wild-type strain at 1 h and 12 h p.i. (Figure 3B and D). This finding indicated that pVir plasmid as a whole does not seem to contribute significantly to the overall process of bacteremia and liver infection caused by *C. jejuni* in our mouse model.

Discussion

C. jejuni is a major cause of sheep abortion worldwide, imposing a significant economic burden for producers. Over the past several years extensive effort has been directed toward the development of *in vivo* model systems suitable to study *Campylobacter* pathogenesis. However, progress toward understanding the pathogenic mechanisms of *Campylobacter* has been hampered by the paucity of high-resolution experimental systems for studying infection *in vivo* (70). These have included studies of primates (9,23,52), dogs (49), pigs (3), chickens (26), ferrets (10), and guinea pigs (17,59). The large number of models speaks to the difficulties encountered in identifying a system that is sufficiently robust to meet research requirements. An optimal animal model for characterizing *Campylobacter* infection should be readily available and reasonable in cost, easy to maintain and handle, and it should able to reproduce the pathology associated with ovine abortion.

Our research group has recently investigated the high virulence of *C. jejuni* clone SA in inducing abortion, and we evaluated its virulence in pregnant guinea pigs (17). Guinea pigs represent a well-established model to study *Campylobacter*-induced abortion (21,58,59), because they are susceptible to *Campylobacter* and the induced disease is similar to that seen in sheep. Although the model is appropriate for investigating *Campylobacter*-induced abortion, it is cost prohibitive (>$300 per animal), time consuming and cumbersome, and is

not feasible for large-scale studies involving multiple experiments. In order to continue our efforts of understanding pathogenesis in detail, an efficient animal model is needed to assess the critical steps involved in *C. jejuni* abortion. Studies have established mouse disease models in which no grossly observable disease occurs following oral challenge, yet dissemination of bacteria to the blood is commonly seen (7). In this study, we determined the unique ability of *C. jejuni* clone SA to produce bacteremia in a mouse model compared with other *C. jejuni* isolates, NCTC 11168 and 81-176. To our knowledge, this work is the first comprehensive study that examines the molecular and genetic factors involved in *C. jejuni*-induced bacteremia using a mouse model following oral challenge.

To develop a reproducible and widely applicable murine model to study *Campylobacter*-induced abortion we chose to use IA3902 an isolate of clone SA, because its pathogenicity has been established and its genome is fully sequenced. We also included non-abortifacient *C. jejuni* strains NCTC11168 and 81-176, originally derived from human clinical isolates, because both strains are also whole genome sequenced and widely used in many research laboratories across the world.

In our preliminary experiments, we determined which mouse strain was more susceptible to systemic infection by clone SA after oral inoculation, and our results clearly demonstrate a discernible difference between the two strains. We initially inoculated commercially supplied BALB/c and CD-1 mouse strains. CD-1 mice and *C. jejuni* IA3902 proved to be the preferred host-parasite combination for studies of pathogenesis, opposed to BALB/c mice, which presented low vulnerability to the organism (Figure 1). CD-1 mice remained persistently bacteremic at a high level until they were euthanized 24h after inoculation. Our inability to efficiently infect BALB/c mice with IA3902 could be

influenced by different factors such as gut microflora, rearing practices or simply the genetic makeup of the mouse (12). With the CD-1 species, we have consistently found that pretreatment with antibiotics is not necessary, in contrast to previous studies (30). With these considerations in mind, we used CD-1 mice.

The pathogenesis of *Campylobacter* spp that induce abortion when acquired by a nonvenereal route of transmission entails oral exposure, intestinal colonization, bacterial invasion, bacteremia, and infection of the fetoplacental unit (20,54-56). The specific mechanisms of bacteremic *Campylobacter* are not well understood. However, the known depression of cell-mediated immunity during pregnancy may be relevant to the development of systemic *Campylobacter* infection and septic abortion (54). In the past, *Campylobacter fetus* subsp. *fetus* was recognized primarily as a cause of systemic illnesses (14), whereas *Campylobacter jejuni* was predominantly recognized as a cause of diarrheal illnesses (66). Blaser et al. (16) reported that *C. jejuni* strains are usually susceptible to the complement-mediated bactericidal activity present in normal human serum, whereas *C. fetus* strains usually are resistant. However, bacteremia attributable to *C. jejuni* has been described (13,38,65), while one study indicated that certain strains of *C. jejuni* have an enhanced ability to induce bacteremia. Additionally, acute hepatitis has been a result of bacteremia triggered by *C. jejuni* in humans (37). In the remainder of the studies, there were no demonstrable clinical signs of infection, despite the bacterial load recovered from the blood and liver tissues. *C. jejuni* IA3902 was remarkably capable in its ability to induce bacteremia following oral inoculation (Figure 3A). Whereas, 81-176 caused minor bacteremia within the first hour, but was unable to survive through 24h in substantial numbers, and 11168 did not induce systemic infection (Figure 3A and B). The fact that both blood and liver tissues

could be readily infected with IA3902 indicates that the mouse model is suitable for assessing the systemic infection by *C. jejuni*. In addition, these results suggest that IA3902 acquired specific traits similar to *C. fetus* when the shift in disease occurred, therefore, allowing it to resist complement-mediated bactericidal activity, and cause bacteremia.

Analysis of the CPS of the *kpsS* mutant by Alcian Blue staining showed that the mutant did not express a capsule. As the CPS mutant still expressed complete LOS molecules (Figure 3), this confirms previous reports that the capsule of *C. jejuni* is expressed independently of the LOS molecule (2).

This study provides the first evidence of a role for the *C. jejuni* clone CPS in pathogenesis. Several *in vitro* studies have indicated that a surface array protein (a capsule-like protein structure) in wild-type *C. fetus* strains plays a critical role in the organism's ability to resist both the complement-mediated bactericidal activity (C3b binding) in normal human serum and phagocytosis by neutrophils (15,16,43). S-plus *C. fetus* strains can apparently penetrate the intestinal wall and produce bacteremia lasting 48-120 h after oral challenge (47). Similar experiments performed on *C. jejuni* 81-176 demonstrated a role for the capsule in serum resistance (5), while more recent studies have revealed that the CPS is required for virulence, and mutations within the kps gene cluster, those that are responsible for transferring the CPS to the outer membrane, showed reduced adherence and invasion of intestinal epithelial cells, and reduced virulence in the ferret model (5). Since the CPS locus of *Campylobacter* is under phase variation, shows high genetic diversity, has the potential to mimic host cell antigens, the ability to resist innate mechanisms such as phagocytosis and complement-mediated killing (32) and the fact that clone SA seems to have a distinct CPS structure (as determined by comparative phenotypic and genomic analyses; unpublished

data), we investigated its role in induction of systemic infection using the mouse model developed in this study. Our observation of the Δ*kpsS* mutant *in vivo* clearly coincides with similar studies confirming *C. jejuni* clone SA CPS is an important bacterial component contributing to bacteremia. The mechanism for this penetration has not been determined, but it is clearly rapid in onset. The inability of the Δ*kpsS* mutant to induce bacteremia highlights the utility of mouse model to assess pathogenesis. Our findings also suggest that the CPS as a whole or an individual component of the CPS contributes to bacteremia similarly to the S-proteins of *C. fetus*. Therefore, the CPS of clone SA must enable the organism to resist complement-mediated activity. This *in vivo* study raises an important question relating to the mechanism by which encapsulated *C. jejuni* clone SA causes disease, how does the CPS enable clone SA to penetrate the intestinal wall and cause systemic infection.

Finally, our mouse model measured the ability of pVir-cured IA3902 to induce bacteremia, and assess if the plasmid plays a role in the induction of systemic infection. Our findings indicated that the pVir plasmid as a whole does not seem to contribute significantly to bacteremia or liver infection (Figure 3B and D). Furthermore, it provides proof that the mouse model is efficient and provides a solid foundation for us to delineate key pathogenic properties associated with clone SA in an inexpensive way.

Collectively, this work has yielded a cost-effective and tractable model system in which critical steps in the pathogenesis of *Campylobacter*-associated abortion can be readily assessed, and provided the first direct evidence for a role of the *C. jejuni* IA3902 CPS in virulence. Besides the future promise of advancing our knowledge of *Campylobacter* pathogenesis, our murine model can potentially identify novel candidates for the development of protective vaccines against *Campylobacter* abortion in sheep.

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Table 1. *C. jejuni* strains used in this study

Strain or Plasmid	Description[a]	Reference
Strain		
C. jejuni		
IA3902	Wild type	(53)
Δ*kpsS* mutant	IA3902 derivative *kpsS:: kan*^{r}	This study
Δ*kpsS* – comp	Δ*kpsS* complemented with pRY112-Δ*kpsS* – comp	This study
81-176	Wild type strain, invasive diarrheal isolate	(27)
NCTC 11168	Wild type strain, non-abortifacient diarrheal isolate	(46)
Plasmids		
pGEM-T	*E. coli* cloning vector	(Promega)
pGEM-T-*kpsS*	pGEM-T containing full-length kpsS, *kan*^{r}	(29)
pMW10	*E. coli-C. jejuni* shuttle vector with promoterless *E. coli* lac Z gene, *kan*^{r}	(67)
pRY112	*E. coli-C. jejuni* shuttle vector, *cat*^{r}	(68)
pRY112- Δ*kpsS* – comp	pRY112 containing the promoter region of *cmeABC* and full-length *kpsS*	This study

[a] *kan*^{r} confers kanamycin resistance; *cat*^{r} confers chloramphenicol resistance.

Table 2. PCR primers used in this study.

Primers	Sequence (5' to 3')[a]
kpsS_F	GCT CAA GTT GAA GAT GAT GCT TCG ATG AT
kpsS_R	CAT ACC AAA ACA GGA TTG GGT TTA TAA GCA TGA
ST101_F	TAG CCG CGG AAA CTT TTA TGC TTA GAA AAA T
ST101_R	CTA GAG CTC TTA GGA TCA TAT CCT GCT ATA T

[a] The underlined sequences in the primers indicate the restriction sites for *SacI* (GAGCTC) and *SacII* (CCGCGG).

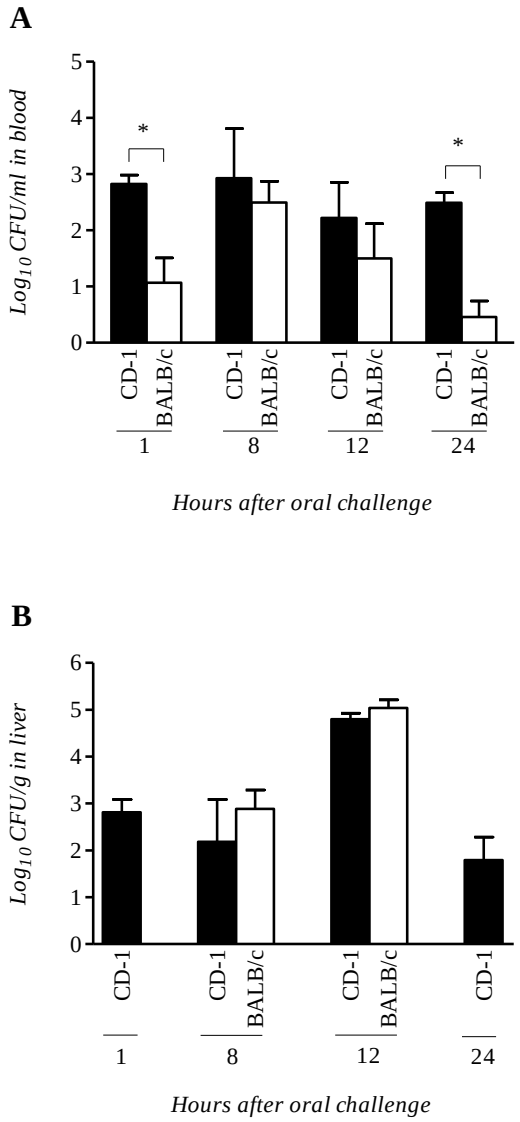

Figure 1. Susceptibilities of selected strains of mice to oral challenge with IA3902 (10^8 CFU). Five CD-1 and 5 BALB/c mice were infected and were necropsied at the indicated hour post-inoculation. Bacteremia (Figure 1A) and liver infection (Figure 1B) was determined as described in materials and methods. Each bar represents the $\log_{10}$ CFU per ml of blood (mean ± SEM) in different strains of mice. *$p < 0.05$ is statistically significant.

‡*liver tissues were only collected at 8 and 12 h p.i. for BALB/c mice, but CD-1 mice liver tissues were collected at 1, 8, 12 and 24 h p.i..*

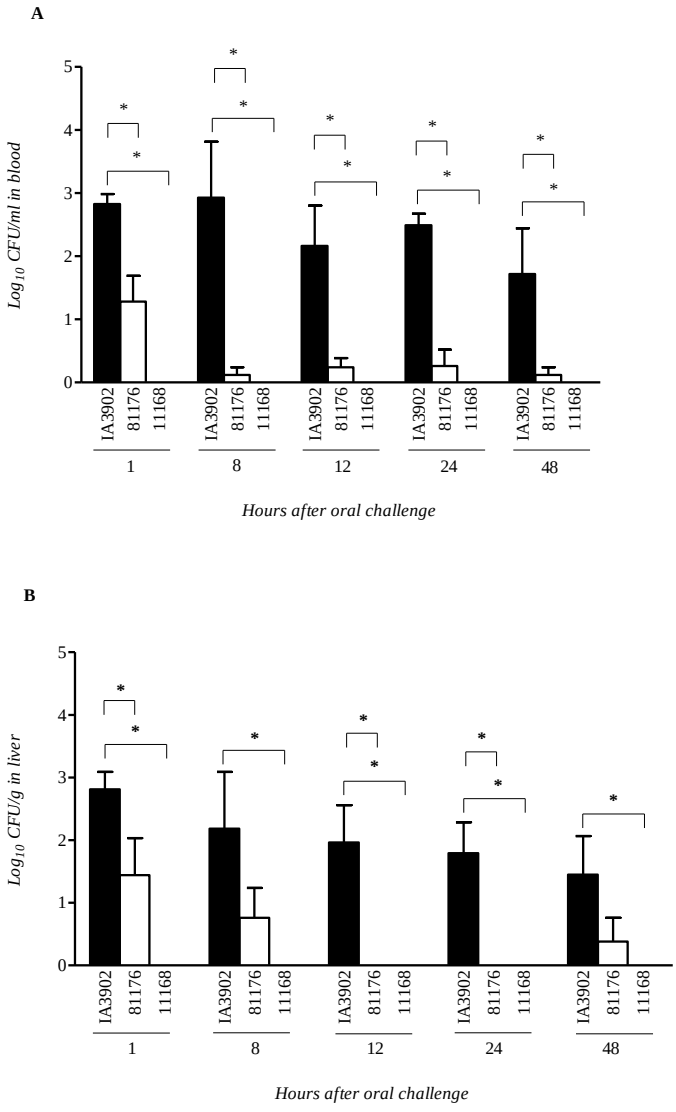

Figure 2. Quantitation of systemic infection by IA3902, 81-176 and NCTC 11168 in CD-1 mice. Mice were orally challenged 10^8 CFU. At each time point, 5 mice were sacrificed and the number of *C. jejuni* in **A)** cardiac blood and **B)** liver tissue was determined. Each bar represents the $\log_{10}$ CFU per ml of blood or liver (mean ± SEM). $*p < 0.05$ is statistically significant. [‡]*Data was collected in different trials; all trials were performed under the same conditions*

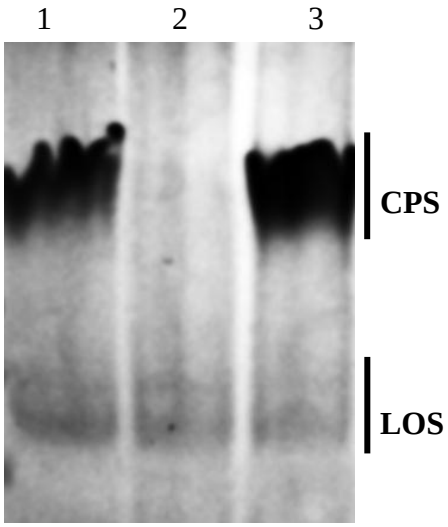

Figure 3. Analysis of capsule expression of *C. jejuni* IA3902 with Alcian Blue staining. **1)** wild-type IA3902 **2)** capsule mutant (Δ*kpsS*) and **3)** complemented mutant (Δ*kpsS*-comp)

Table 3. Motility assay

Strain	Diameter (*mm*) (mean ± SEM)
IA3902	78±0.58
Δ*kpsS* mutant	80±0.33
Δ*kpsS* – comp	80±0.88

Fresh bacterial culture grown < 24h was harvested and diluted with MH broth to the desired optical density. 5µl of culture was stab-inoculated into motility agar plates and incubated for 48 h under standard conditions for *Campylobacter*. The Δ*kpsS* mutant and Δ*kpsS* – comp retained the wild-type level of motility.

Table 4. Ability to grow on selective media

	Log_{10} CFU/mL (mean ± SEM)	
Strain	MH growth	MH + SS growth[a]
IA3902	1.7±0.060	1.9±0.044
Δ*kpsS* mutant	1.7±0.090	1.6± 0.15
Δ*kpsS* – comp	1.4±0.060	1.4±0.18

[a] SS denotes *Campylobacter* selective and growth supplements media.
Fresh bacterial culture grown 18 h was harvested and diluted with MH broth to the desired concentration based on optical density, followed by serial dilutions, plated on MH agar plates or MH+SS agar plates and incubated for 48 h under microaerobic conditions.

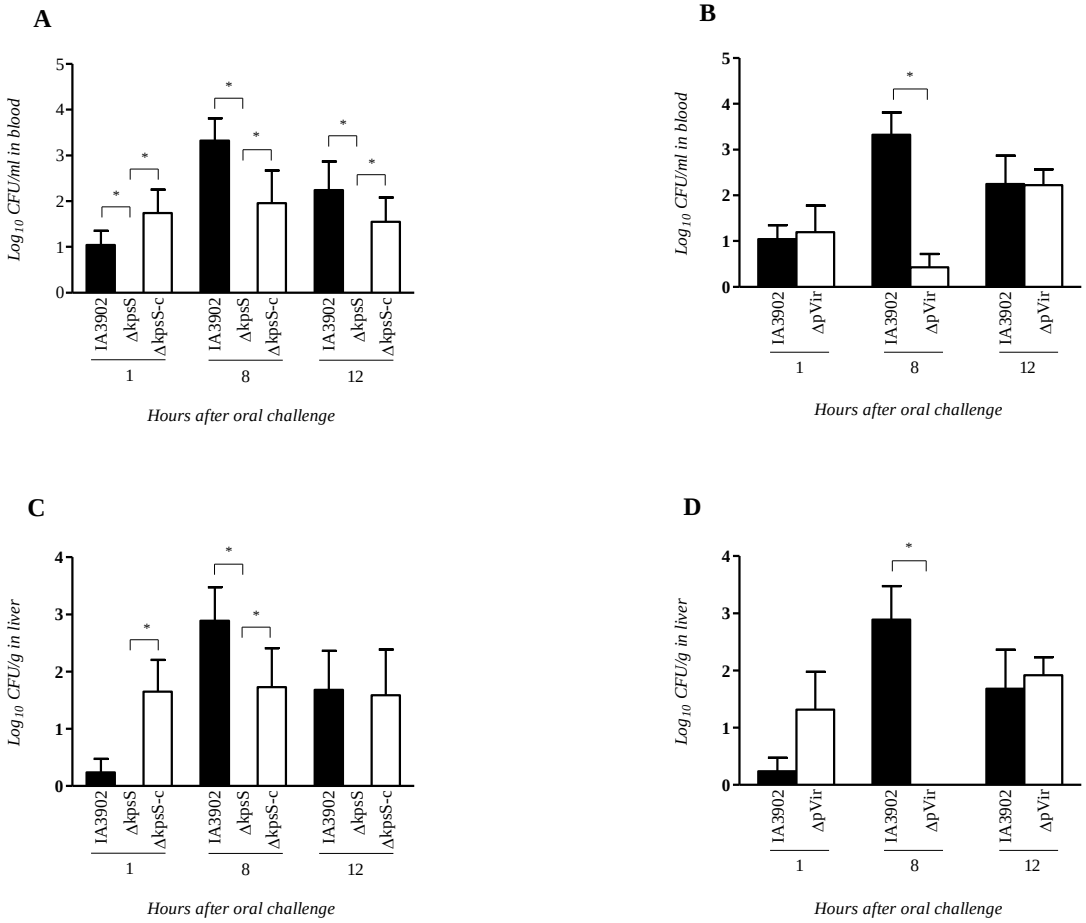

Figure 4. Effect of CPS (A, C) and pVir (B, D) on systemic infection of mice by *C. jejuni.* CD-1 mice (n=8/group) were challenged with 10^8 CFU via gastric gavage of either wild-type IA3902, Δ*kpsS*, Δ*kpsS*-comp or pVir mutant and CFUs were determined post-infection at different time points. Each bar represents the $\log_{10}$ CFU per ml of blood or liver (mean ± SEM). $*p < 0.05$ is statistically significant. *(Data was collected in different trials; all trials were performed under the same conditions)*

CHAPTER 3. GENERAL CONCLUSION

Campylobacter infection is one of the major causes of ovine abortions worldwide. Our work recently demonstrated the emergence of a predominant *C. jejuni* clone SA associated with sheep abortion in the United States. In addition to causing sheep abortion, *C. jejuni* is among one of the leading causes of bacterial foodborne disease in developed countries. However, as a zoonotic pathogen, little is known about the pathogenic mechanisms and the factors that influence the emergence and transmission of this highly pathogenic clone. Therefore, understanding the nature, regulation, and mechanisms of action are indispensable for prevention and treatment of clone SA among sheep and controlling the transmission to humans.

In this study, we developed a mouse model that is suitable for assessing bacteremia, a key element in *Campylobacter*-associated abortion and revealed genetic mechanism(s) that contribute to bacteremia.

Our results indicated that clone SA is highly pathogenic in orally challenged CD-1 female mice and has the ability to disseminate quickly and induce bacteremia. In contrast, the non-abortifacient *C. jejuni* strain NCTC 11168 and *C. jejuni* 81-176 were incapable of producing bacteremia or produced substantially lower levels of bacteremia, respectively. These findings clearly indicate that the SA clone has acquired unique virulence properties, similar to those of *C. fetus* allowing it to resist complement-mediated bactericidal activity, in addition, this mouse model will provide us with the appropriate means to investigate how pathogenic *Campylobacter* cause systemic infection in infected animals.

Generating an insertional mutation in the *kpsS* gene, a capsule transporter protein, completely knocked out the expression of the CPS in *C. jejuni* IA3902, as indicated by Alcian Blue staining, while complementation fully restored the expression of the CPS. Oral inoculation of CD-1 mice with Δ*kpsS* IA3902 mutant strain did not induce bacteremia, identifying CPS a major virulence factor of the SA clone. These findings suggest that clone SA has potentially acquired CPS structures similar to the surface layer proteins of *C. fetus*, which have historically been associated with bacteremia and systemic infection. Despite its perceived virulence potential, the pVir plasmid, that is not unique to clone SA, was not found to contribute significantly to bacteremia, suggesting that pVir is not a major factors involved in producing systemic infection in our mouse model.

Together, these results will advance our understanding of the pathogenic mechanisms involved in systemic infection due to *Campylobacter*, allow us to investigate more genetic factors that may contribute to bacteremia and will aid in the development of means to control this important disease in animals.

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